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Study of vesicle leakage induced by melittin

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Abstract

The leakage induced by melittin, a membrane-perturbing amphipathic peptide, from large unilamellar 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) vesicles was studied using calcein as fluorescent marker. The extent of leakage has been found to be regulated by the melittin/lipid molar ratio. Melittin leads to the complete release of trapped calcein from some vesicles. This all-or-none mechanism leads to the co-existence of two different vesicle populations: the 'empty' and the intact one. Intervesicular migration of melittin was not observed. The results reveal a specific targeting of the lysed vesicles by melittin. The presence of negatively charged lipids (unprotonated palmitic acid or 1-palmitoyl-2-oleoylphosphatidylglycerol) in the neutral POPC matrix inhibits the lytic power of melittin; this inhibition increases with increasing surface charge density. It is proposed that the anchorage of the peptide on the charged surface prevents the formation of defects allowing leakage. A statistical model based on a random distribution of the peptide molecules on the vesicles is proposed to describe the release induced by melittin. It is proposed that about 250 melittin molecules per vesicle are required to affect the bilayer permeability and to empty a vesicle of its content. This large number suggests that leakage is more likely due to collective membrane perturbation by the peptide rather than to the formation of a well-defined pore.

Keywords: Melittin; Vesicle leakage; Permeability; Calcein; Lipid; Lipid–peptide interaction

1. Introduction

Several classes of molecules such as simple surfactants or amphipathic peptides perturb membrane permeability and induce the release of trapped material [1,2]. Their presence can lead to the release of haemoglobin from erythrocytes, causing the death of the cell, or the release of a drug from a liposomal preparation. Knowledge of the precise mechanism responsible for permeability perturbation is required in order to understand and/or control the lytic activity of these molecules. In this paper, we have studied the mechanism by which melittin, an amphipathic peptide, induces the release of trapped material from phosphatidylcholine (PC) vesicles. This peptide is a relevant model for studying the mechanism of release since its lytic activity shares common features with other cytotoxic agents including complement [3,4]. We have also characterized the role played by electrostatic interactions in the content release.

Melittin is a popular model peptide known for its strong interaction with membranes (for review see [5]). This peptide, constituted of 26 amino acids with 5–6 positive charges, binds to membranes as an amphipathic α-helix [6]. Melittin increases the permeability of biological and model membranes [7–12]; this issue is not a matter of debate. Nevertheless, the dynamic process responsible for the changes in permeability properties is still unknown. DeGrado et al. [8] found that haemoglobin release from human erythrocytes exhibits a biphasic time course. It is proposed that rapid haemolysis due to melittin-induced transient openings is followed by gradual haemolysis produced by internalized melittin dimers. It is concluded that all erythrocytes were partially lysed. Conversely, Tosteson et al. [7] have proposed that, in isotonic NaCl, melittin-induced haemolysis is due to transient openings in the membrane leading to complete lysis of some cells whereas others remain intact. A colloid osmotic mechanism is proposed in which the release of haemoglobin follows the formation of ion channels in the membrane leading to osmotic swelling of the cells. Recently, Portlock et al. [12] investigated melittin-induced permeability changes on ery-

Abbreviations: EDTA, ethylenediaminetetraacetic acid; LUV, large unilamellar vesicle; Mel, melittin; PA, palmitic acid; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; PS, phosphatidylserine.

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throcytes and large lipid vesicles. The potency of melittin to induce membrane perturbation is different for erythrocytes and pure lipid bilayers. They conclude that this might be due to melittin–protein interactions in the erythrocyte membrane. From kinetic measurements on small unilamellar 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) vesicles, Schwarz et al. [11] infer that melittin forms pores through which the depletion occurs. They suggest that the rate of pore formation depends on melittin concentration and that the rate-determining step is the reversible dimer formation. However, others [13,14] suggest that increases in permeability are not due to channel formation, but to peptide-induced disordered region in the bilayer. Accumulation of peptide on the membrane surface decreases its stability and enhances the permeability.

In this study, we have sought to get more insight into the mechanism by which melittin induces the release of entrapped dye from large unilamellar vesicles (LUVs). The first question that we addressed concerned the pathway of the release. We have established whether the release follows an all-or-none pathway (a fraction of the vesicles looses their entire content and the other remains intact) or a gradual release (all the vesicles release a fraction of their content). Second, we have characterized the dependence of the content release on melittin/lipid ratio. These results allowed us to develop a model based on simple statistics to relate the melittin/lipid ratio and the extent of content release. Electrostatics of positively charged melittin may play an important role in controlling the release. Thus, we investigated the modulation of the permeability properties related to changes in charge density at the surface of the vesicles.

The experimental technique made use of the self-quenching properties of calcein, a fluorescent marker [15]. Its release from the vesicles can be monitored directly by increasing fluorescence intensity. The term lysis originally used to describe cell haemolysis but later used for all kinds of morphological membrane perturbations, refers, in this paper, to the release of trapped calcein.

2. Materials and methods

2.1. Materials

POPC and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). Palmitic acid (PA) was obtained from Sigma (St. Louis, MO, USA). Melittin was purified from bee venom (Sigma) by ion exchange chromatography on SP-Sephadex C-25, and desalted according to the high-performance liquid chromatography procedure described in Lafleur et al. [16]. Calcein (2,4-bis-(*N*,*N*′-di(carboxymethyl)aminomethyl)fluorescein) was purchased from Molecular Probes (Eugene, OR, USA) and used without further purification.

2.2. Preparation of vesicles

A stock solution of each lipid was prepared in benzene. The lipid mixtures were obtained by mixing appropriate volumes of the stock solutions. Lipids were lyophilised from benzene and then hydrated with a dye-containing borate buffer (80 mM calcein, 150 mM H_2BO_3, 5 mM ethylenediaminetetraacetic acid (EDTA), 20 mM NaCl, 5 mM KOH, adjusted to pH 9 with 5 M NaOH) to give a liposomal suspension of approx. 10 mM. The lipid suspension was then freeze-thawed five times from liquid nitrogen to room temperature. Subsequently, the dispersion was extruded ten times through two stacked polycarbonate filters of 100 nm pore size (Nucleopore) to obtain LUVs. The calcein-containing vesicles were separated from the free calcein by exclusion chromatography using a column filled with Sephadex G-50 fine gel swollen in an isotonic buffer (150 mM H_2BO_3, 5 mM EDTA, 140 mM NaCl, 5 mM KOH, pH 9). The dilution factor on the column was about 3, resulting in an approximate phospholipid concentration of 3 mM for the eluted vesicles. The precise phospholipid concentration was determined according to the Fiske-SubbaRow phosphorus assay [17].

2.3. Leakage experiments

The LUV dispersion was diluted with isotonic buffer in order to obtain a phospholipid concentration of 6 μM in the cuvette. This dilution ensured a dye concentration less than 1 μM after total release from all the vesicles. Our standard curve describing the fluorescence intensity as a function of calcein concentration showed that the fluorescence intensity of calcein is directly proportional to its concentration up to 1 μM. The high concentration (80 mM) of the encapsulated marker led to self-quenching of its fluorescence, resulting in low background fluorescence intensity of the vesicle dispersion (I_B). Afterwards, an aliquot of melittin solution was added to the stirred dispersion in order to obtain the desired peptide/lipid ratio. It should be noted that under our conditions, melittin is predominantly in the monomer form [18]. Three controls have been done to verify effective stirring. First, the measured leakage showed no dependence on stirring speed beyond the speed we used. Second, the concentration of melittin solution added to the vesicle dispersion was 1 μM. With significantly more concentrated melittin solution, the leakage curves were not reproducible. Third, if the vesicles were added to melittin already diluted in the cuvette, the same extent of release was observed, confirming the effective stirring. Release of calcein caused by the addition of melittin led to the dilution of the dye into the medium and could therefore be monitored by increasing fluorescence intensity (I_F). The experiments were normalized relative to the total fluorescence intensity (I_T) corresponding to the total release after complete disruption of all the vesicles by Triton X-100 (0.1 vol%). The results of

the leakage experiments are presented as the percentage of released calcein, calculated according to:

$$\% \text{ release} = 100(I_F - I_B)/(I_T - I_B) \quad (1)$$

The reproducibility of these experiments for a certain lipid/melittin incubation ratio is ±5%.

2.4. Mechanism assays

Using a three step experimental procedure, we investigated the mechanism of melittin-induced release. The first step was the determination of a calibration curve for fluorescence self-quenching efficiency of vesicle-entrapped calcein. A series of POPC vesicles were prepared in buffers containing various calcein concentrations: 5, 10, 25, 50, 80 and 100 mM. After the removal of non-entrapped calcein on a Sephadex G-50 column, an aliquot of each vesicle dispersion was diluted (final lipid concentration 6 μM) and its fluorescence intensity measured; this value corresponds to the fluorescence of vesicle-entrapped calcein (I_B). The total fluorescence intensity was determined after complete disruption of the vesicles with Triton X-100 (I_T). The self-quenching efficiency (Q) for each calcein concentration could be calculated according to:

$$Q = (1 - (I_B/I_T)) \times 100 \quad (2)$$

The second step was the determination of the percentage of calcein release after incubation with melittin. A vesicle population with an entrapped calcein concentration of 80 mM was prepared with a final phospholipid concentration of 3 mM. Different amounts of melittin were added to 100 μl aliquots of LUVs. A 5 μl aliquot from every mixture was withdrawn, diluted in 2.5 ml isotonic buffer and its fluorescence measured (I_F). The total fluorescence (I_T) from this sample was obtained after addition of Triton X-100. The background intensity (I_B) was determined using a blank without melittin. The percentage of release was calculated according to Eq. (1). The last step was the determination of the self-quenching efficiency of entrapped calcein after incubation with melittin. The same amount of melittin as used in the second step was added to a 100 μl aliquot of the same LUV dispersion. After incubation with melittin, the sample was passed down a Sephadex G-50 column to remove released calcein. The portion containing the vesicles was collected, diluted and its fluorescence measured before and after addition of Triton. The self-quenching efficiency was calculated according to Eq. (2). Control experiments have shown that the self-quenching efficiency was independent of the lipid concentration up to 10 μM lipid and therefore needed no correction for differences in lipid concentration.

2.5. Spectroscopic method

Fluorescence measurements were performed on a SPEX Fluorolog-2 spectrometer. The fluorescence intensity of calcein was monitored using an excitation wavelength of 490 nm, an emission wavelength of 513 nm and a response time of 0.5 s. The excitation and emission bandpath widths were set at 1.3 and 0.5 mm, respectively. The spectrometer was equipped for sample stirring and temperature control.

3. Results and discussion

3.1. Melittin-induced calcein release from vesicles

The influence of melittin on the permeability of phospholipid bilayers was studied using the self-quenching properties of calcein. We present a typical measurement showing a curve which can be divided into three sections (Fig. 1A). The first part is the background intensity (I_B) of the entrapped calcein in self-quenching concentrations. As shown in the second part of the curve, the addition of melittin to the vesicles causes the leakage of the marker, leading to increasing fluorescence intensity (I_F). Total fluorescence intensity (I_T) is determined by the complete disruption of all vesicles by Triton X-100. An important feature of this curve is the fact that the fluorescence intensity reaches a plateau few minutes after the addition of melittin. Even after a 30 min incubation, the fluorescence intensity does not change significantly once the plateau is reached. This indicates that no further release occurs after a certain period. An equivalent release profile has been observed for melittin-induced leakage of carboxyfluorescein from small unilamellar vesicles [11] and of haemoglobin from red cells [7]. Several amphipathic peptides, including gramicidin S, alamethicin [12] and α-haemolysin [19], show also a similar release profile. This behaviour implies that melittin binds rapidly to the vesicles and does not exchange between vesicles. First, the rapid binding has already been confirmed by a kinetics study showing that the rate of binding of melittin to lipid vesicles was in the order of ms [10]. This contrasts with the results obtained with a fragment of pardaxin, another membrane-perturbing peptide; in that case, the peptide binds slowly to membranes and leads to a release of trapped material occurring over more than 15 min [20]. The fast binding of melittin is therefore a prerequisite for the stabilization of the fluorescence intensity after few minutes. Second, we have investigated the absence of melittin migration between vesicles and of significant fraction of free melittin by adding a second population of intact vesicles to a first completely lysed vesicle population (Fig. 1B). For the first part of the experiment, we added melittin to the vesicles in the minimal concentration ensuring total lysis (melittin/lipid ratio of $90 \cdot 10^{-4}$). A second aliquot of intact vesicles is then added, giving a melittin/lipid ratio of $45 \cdot 10^{-4}$ in the cuvette. For such a sample, a release of at least 75% is expected. If melittin migrated between the vesicles or if there were a significant amount of free melittin, the intact vesicles would release

their content, giving rise to increasing fluorescence intensity. The results show no significant increase in fluorescence intensity after the addition of the second vesicle aliquot (the small increase corresponds to the incomplete self-quenching of trapped calcein). The overall release is only 48%. This indicates the strong binding of melittin to the already attacked vesicles and the absence of significant amount of free melittin. Similar behaviour has also been reported for the haemolysis of erythrocytes [8]. This phenomenon is in agreement with the high binding constant of melittin to phosphatidylcholine [21–23], indicating that, in our conditions, only a minute proportion of melittin is free in solution. A very slow dissociation kinetics, supported by the same type of experiments, has been also proposed for

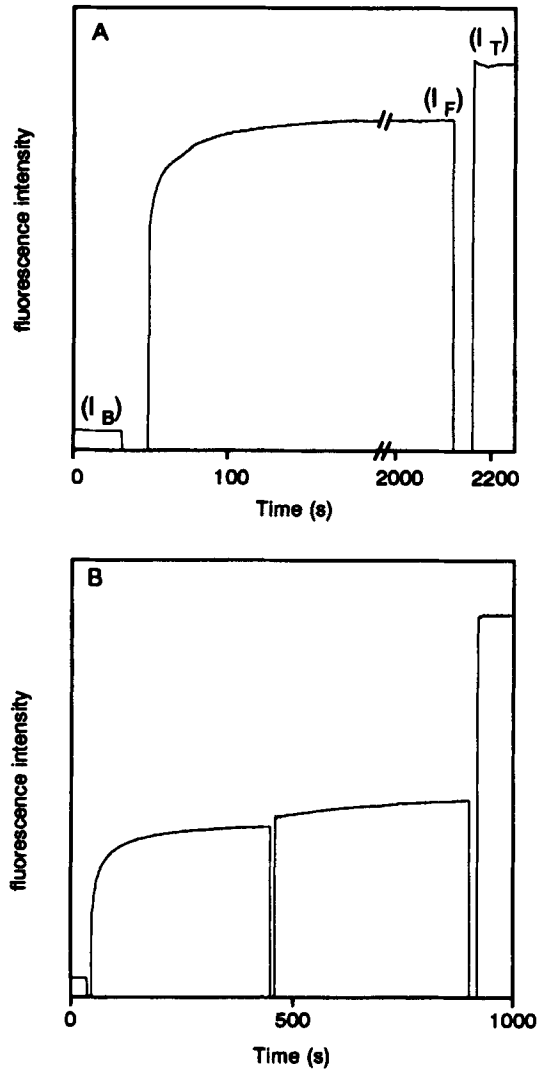

Fig. 1. Calcein release induced by melittin. (A) (I_B) Background intensity of the calcein encapsulated in POPC vesicles, (I_F) Melittin-induced release leads to dilution of the dye into the medium and increasing fluorescence intensity (Mel/POPC ratio of 1:160), (I_T) Total fluorescence intensity after addition of Triton. (B) Addition of melittin to POPC vesicles leading to their complete release (Mel/POPC ratio of 1:110), and a subsequent addition of an intact vesicle aliquot (final Mel/POPC ratio of 1:220).

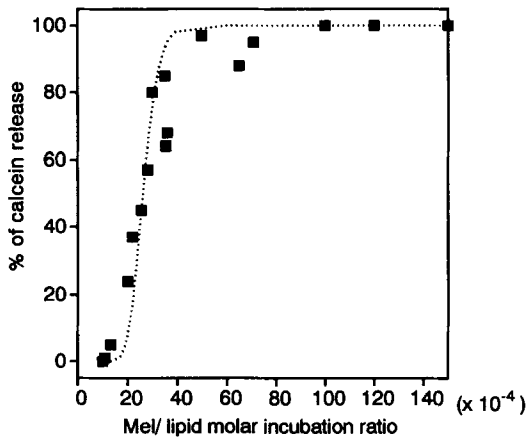

Fig. 2. Percentage of calcein release from POPC vesicles as a function of melittin/POPC molar incubation ratio. The dashed line was calculated according to the statistical model leading to Eq. (4).

HDL apolipoproteins [24]. This behaviour contrasts with that observed for bacterial toxin α-haemolysin [19]. In the case of that toxin, the addition of a second population of intact vesicles leads to increasing fluorescence. This phenomenon has been suggested to be associated to a low membrane/water partition coefficient of α-haemolysin; the amount of free toxin in solution should be high enough to induce the leakage of the second vesicle population. In this regard, a detectable calcein release is observed when a second aliquot of intact vesicles is added to a sample containing melittin in a concentration significantly higher than that required to get 100% release from the first vesicle aliquot (a melittin/lipid molar ratio of $500 \cdot 10^{-4}$) (data not shown). In such conditions, it is likely that the amount of free melittin is sufficient to induce leakage from the intact vesicles. The results presented in Fig. 1 indicate that melittin added to lipid vesicles binds rapidly, almost completely, and without subsequent intervesicular migration.

The quantity of released calcein depends on the molar incubation melittin/POPC ratio (Fig. 2). A total release is induced by melittin/POPC incubation ratios greater than $90 \cdot 10^{-4}$. The curve also shows that a considerable amount of melittin (a melittin/POPC ratio of $10 \cdot 10^{-4}$) can be added without inducing detectable release. The amount of melittin required to induce the release of trapped material is in the same order than that previously reported for eggPC vesicles [12].

3.2. Mechanism of the lysis

The self-quenching properties of calcein can be used to design experiments to test whether melittin-induced release of calcein from vesicles is a gradual or an all-or-none release. For a gradual release, the calcein concentration in all the vesicles decreases proportionally to the percentage of lysis. Conversely, the all-or-none pathway leads to two different vesicle populations: the intact vesicles with cal-

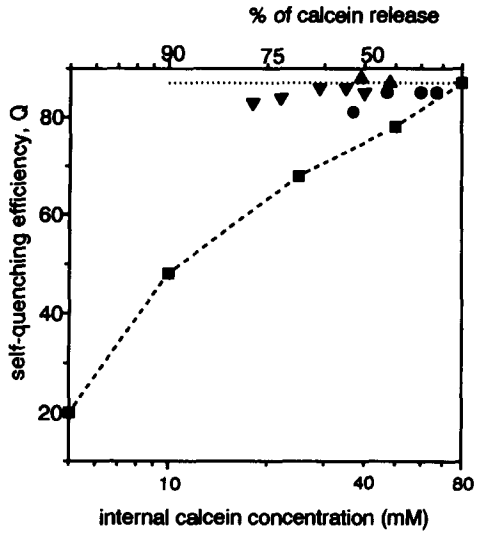

Fig. 3. (■) Self-quenching efficiency of calcein entrapped in POPC vesicles as a function of internal calcein concentration (lower x-axis). Self-quenching efficiency as a function of calcein release (upper x-axis) (▼) for POPC and POPC containing (▲) 10 mol% PA or (●) 10 mol% POPG.

cein trapped at its initial concentration and the empty vesicles having released entirely their calcein content. These two mechanisms can be distinguished by the measurement of the self-quenching efficiency of trapped calcein since it depends on the fluorophore concentration. A gradual release can be identified by decreasing self-quenching efficiency whereas the self-quenching efficiency of the calcein for the all-or-none pathway remains at the initial value. Fig. 3 illustrates the self-quenching efficiency of a series of vesicle dispersions as a function of vesicle-entrapped calcein concentration (lower x-axis) and the self-quenching efficiencies measured after incubation with melittin (upper x-axis). The results indicate clearly an all-or-none pathway for melittin-induced lysis of POPC vesicles since the self-quenching efficiency obtained for different percentages of release is constant. It should be mentioned that freeze-fracture experiments have shown that fluid lipids form large unilamellar vesicles in the presence of melittin at a melittin/lipid ratio as high as $8000 \cdot 10^{-4}$ [25]. In the present study, the proportion of melittin is at least 4-times smaller; it is likely that all the lipids exist under a vesicular form after the action of the toxin. Introducing negatively charged lipids as unprotonated palmitic acid or POPG into the neutral phospholipid matrix does not change the mechanism of melittin-induced release. This finding is in agreement with the all-or-none mechanism previously observed for melittin-induced lysis of small unilamellar vesicles [11]. In addition, experiments with melittin on erythrocytes also result in two cell populations: some cells were completely lysed, whereas the others kept their initial haemoglobin content [7]. An all-or-none pathway has also been observed for HDL apolipoproteins [24] and several other toxins including α-haemolysin [19], GALA [26], magainin 2a [27] and granule cytolysin [28]. The all-or-none pathway reported for all these membrane-perturbing amphipathic peptides suggests that a common mechanism may be at the origin of the release of trapped material.

3.3. Attraction of melittin to lysed vesicles

As discussed in the previous section, the melittin-induced release of trapped material leads to two different vesicle populations: an empty and an intact one. These populations likely differ in other aspects than their content. In order to highlight other properties that distinguish the two populations, the specific targeting of one population by melittin was examined. We investigated whether melittin added to a sample containing these two populations distributes equally or exhibits a specific affinity for one of them. In the case that melittin cannot distinguish between empty and intact vesicles, the percentage of calcein release should be dependent only on the amount of added melittin; it should be the same whether a certain amount of melittin is added to the vesicles in one or N fractions. However, if melittin is specifically attracted to one population, the percentage of release will depend on the number of fractions, N. If melittin targets specifically the empty vesicles, the calcein release should decrease with N. In this case, more melittin than the proportion corresponding to a random distribution would go onto already lysed vesicles, leaving, as a consequence, less melittin to induce the release. Conversely, if melittin targets specifically the intact vesicles, calcein release should increase with N. The results presented in Fig. 4 address the question of melittin ability to distinguish between the two populations. In Fig. 4A, we compare the percentage of release obtained when melittin is added to POPC vesicles in a melittin/lipid ratio of $60 \cdot 10^{-4}$, melittin being added in 1, 2, 3 or 4 fractions. The results clearly show the decreasing extent of release observed with increasing N. This indicates that melittin can distinguish between the two vesicle populations and has a preferential affinity for the empty one. This conclusion is confirmed by the results of Fig. 4B. In this case, we added five aliquots containing $15 \cdot 10^{-4}$ melittin per POPC to the vesicles. The percentage of release was measured after every addition. Knowing the melittin/lipid ratio after every addition, we determined by linear intrapolation the percentage of release from the data presented in Fig. 2; these points reflect the calcein release measured when melittin is randomly distributed onto the vesicles – random distribution is ensured by the addition of melittin in a single aliquot and by effective stirring. Comparison between the two curves shows that the percentage of release obtained for a random distribution is higher than that obtained when multiple aliquots are added; the difference is more pronounced with increasing N. This clearly demonstrates again that melittin shows a specific affinity for empty vesicles.

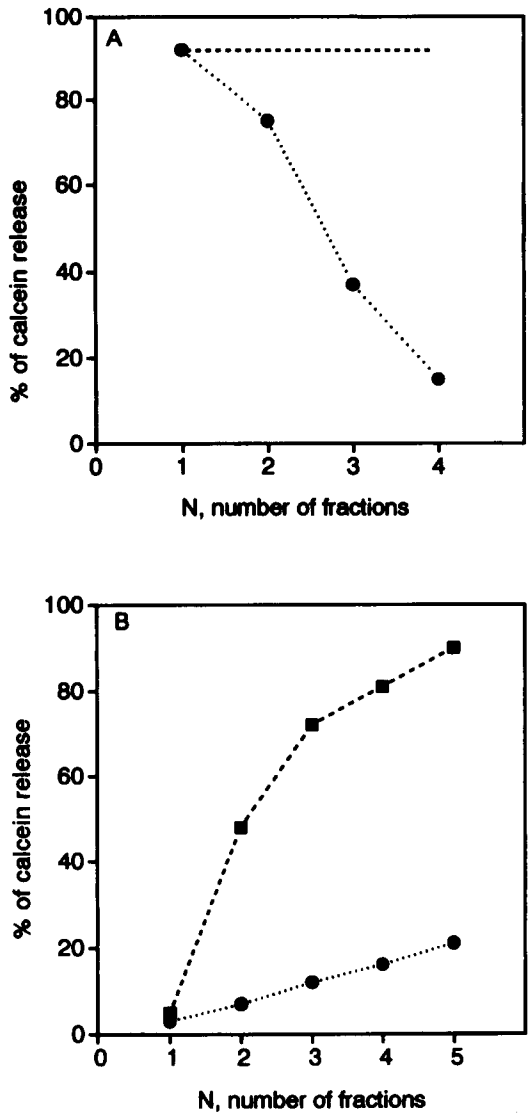

Fig. 4. Dependence of calcein release on the number of melittin fractions, *N*. (A) Melittin is added to POPC vesicles to obtain a final melittin/lipid ratio of $60 \cdot 10^{-4}$ in each case. Melittin was added in different number of fractions. The dashed line represents the expected behaviour for a random distribution of melittin on the vesicles. (B) Melittin aliquots corresponding to $15 \cdot 10^{-4}$ melittin/lipid were added to POPC vesicles. (●) Calcein release measured after the addition of each aliquot to the same sample. (■) The corresponding % release linearly intrapolated from the data in Fig. 2 (i.e., corresponding to a single addition).

These important results reveal that the two vesicle populations are such that melittin can target preferentially the lysed vesicles. We can suggest few phenomena at the origin of this targeting. At a molecular level, it has been shown that melittin slightly decreases the order of the lipid acyl chains [29,30]; this disordering can ease the interaction of melittin with empty vesicles. The presence of melittin also affects the properties of lipid bilayers. The disordering mentioned above should lead to bilayer thinning. It is also proposed that melittin modulates the surface curvature of membranes [31]. Such changes can be at the origin of the specific targeting. Finally, the lipid vesicles are initially in isosmotic conditions. However, after the release of calcein, the conditions are not well defined anymore for the leaky vesicles. The role played by osmotic gradients in melittin-induced lysis is presently investigated in our group. It should be noted that the binding of melittin at the surface of the neutral phospholipid matrix leads to a net positive surface charge density. The repulsive electrostatic interactions between the surface and the melittin molecules in solution do not appear to play an important role; this is consistent with previous results showing that these repulsive interactions play a negligible role in the partitioning kinetics at high salt concentration [23].

The targeting of the empty vesicles by melittin has not been observed for other amphipathic peptides. Both α-haemolysin [19] and GALA [26] induce leakage of vesicles by an all-or-none mechanism. However, the final extent of leakage has been shown to be independent of the number of fractions, suggesting no preferential interaction with a type of vesicles (intact or empty). At this point, we have not identified the reasons how melittin can distinguish between empty and intact vesicles, whereas α-haemolysin and GALA cannot; a better characterization of the differences between the two populations has to be done in the first place.

3.4. Modulation of the release by charged lipids

We have investigated if the lipid composition of the vesicles may allow us to control the melittin-induced release of calcein. Because melittin is a basic peptide carrying 5–6 positive charges, electrostatics should play an important role for peptide–lipid interactions. Therefore, calcein release has been measured for vesicles with different surface charge density. We introduced negatively charged lipids as unprotonated palmitic acid (PA) or phosphatidylglycerol (POPG) in the zwitterionic POPC matrix. Basic pH has been used to ensure complete deprotonation of the fatty acid; its pK_a is estimated to 8.7 [32]. Comparison of the lytic power of melittin between neutral and negatively charged surfaces is presented in Fig. 5A and B. In spite of the favourable electrostatic interaction between the positive amphipathic peptide and the negatively charged bilayer surface, the presence of unprotonated palmitic acid or POPG limits the release of calcein. The inhibition of the lytic power of melittin is proportional to the quantity of negatively charged lipid, in the range investigated. For example, a melittin/lipid molar incubation ratio of 1:300 is required to induce approx. 60% of calcein release for pure POPC. Introducing 50% of POPG, three times more melittin (melittin/lipid ratio of 1:100) is required to induce an equivalent release. A similar protective effect has been observed for phosphatidylserine (PS) [12]. The melittin/lipid molar ratio for 50% release shifts from $33 \cdot 10^{-4}$ for eggPC to $66 \cdot 10^{-4}$ for eggPC/PS 85:15. The inhibition of the lytic activity of melittin appears to be a general feature of the negatively charged lipids.

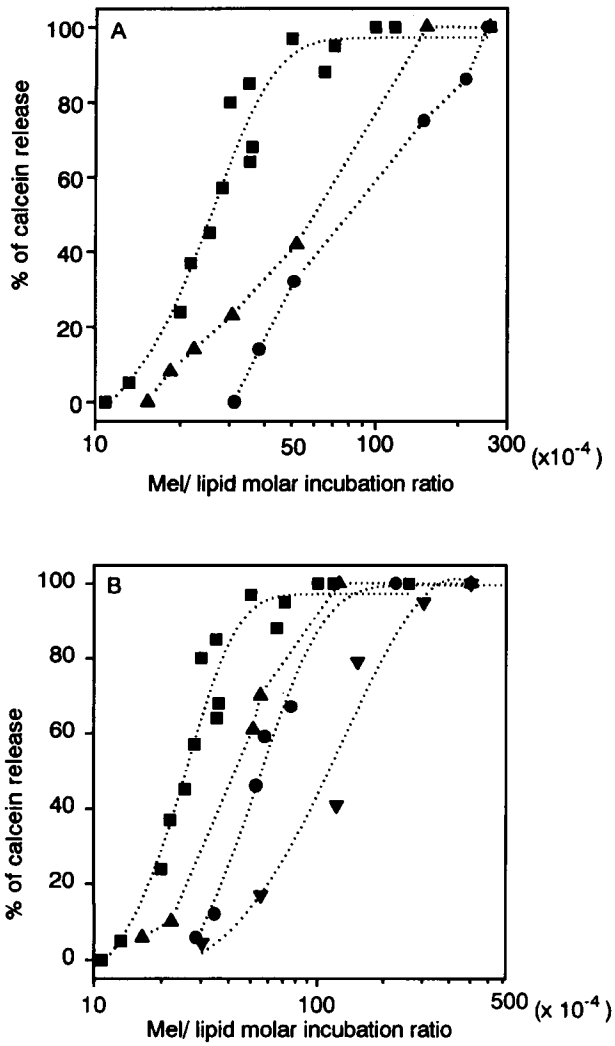

Fig. 5. Effect of increasing negative charge density on melittin-induced release of calcein trapped in vesicles formed with (A) (■) POPC, (▲) POPC/15 mol% PA, (●) POPC/30 mol% PA, and (B) (■) POPC, (▲) POPC/10 mol% POPG, (●) POPC/30 mol% POPG and (▼) POPC/50 mol% POPG.

Several studies have shown that association of positively charged melittin with lipid bilayers is enhanced by negative charges [33,34]. Thus, the inhibition is not caused by a reduced binding of the peptide to the bilayer. It has been shown that the power of melittin to induce micellization of multilamellar phosphatidylcholine bilayers decreases in the presence of negatively charged lipids [35]. To rationalize this behaviour, two key steps of the micellization have been highlighted: the binding of melittin to the bilayer interface and its redistribution leading to micellization. The inhibition of the lysis due to anionic lipids has been proposed to be associated with the anchorage of the peptide at the bilayer surface via electrostatic interactions, preventing its redistribution in the bilayer and, as a consequence, the disruption of the membrane. A similar argument can be made to explain the inhibition of calcein release. Melittin anchored on the surface through electrostatic interactions cannot penetrate deeply enough into the membrane to induce a leak. This phenomenon could be at the origin of the 'non-lytic binding sites' proposed in [12]. A similar two step approach has been suggested to rationalize the permeability perturbations induced by magainin-1 [2].

The inhibition of the leakage by negatively charged lipids has been observed for nisin, a positively charged amphipathic antibiotic. It has been reported that the presence of anionic lipids in dioleoylphosphatidylcholine vesicles reduces the leakage induced by nisin [36]. However, the opposite effect is observed for magainin-1: the presence of negatively charged lipids in eggPC increases the lytic power of this toxin [2]. This set of results indicates that the influence of negatively charged lipids on the lytic properties of cationic peptides is dictated by the affinity of the peptide for zwitterionic membranes. In the case of melittin which interacts spontaneously with phosphatidylcholine bilayers, a negative surface charge density of the bilayer restricts the mobility of the peptide and inhibits vesicle micellization and leakage. Conversely, magainin-1 appears to have a weak interaction with zwitterionic bilayers; a negative surface charge density of the bilayer increases the affinity of the peptide for the lipid bilayer and, as a consequence, its lytic power. It should be noted that the lytic power of melittin is considerably greater than that of magainin. A total release is observed with melittin/lipid ratios greater than $90 \cdot 10^{-4}$, whereas magainin/lipid ratios causing total release are greater than $800 \cdot 10^{-4}$ [2].

3.5. Statistical model describing the release

A simple statistical model is proposed to rationalize melittin-induced release. The interpretation of this type of experiments must not assume a uniform distribution of the lytic agent on the vesicles; actually, it is unlikely that the same number of melittin molecules is bound to each vesicle. The basic assumption of our approach is the random distribution of melittin onto the vesicles; this means that the j^{th} melittin can go randomly onto the i^{th} vesicle. The random distribution can be described by a Gaussian distribution because of the large number of vesicles and the high mean number of melittin per vesicle. Melittin/lipid incubation ratios can be expressed as the number of melittin per vesicle, M, assuming that a 100 nm vesicle is formed by about 10^5 lipids (the lipid molecular area is estimated to 68 Å^2 [37]). M can vary between 0 and the maximum number of melittin that a vesicle can bound. For simplicity, this latter value is approximated to infinity since, in our conditions, the probability of having a vesicle with more than 25% of its total area covered by melittin is negligible. Taking into account the fact that some vesicles are completely lysed whereas others are still intact, the concept of an apparent threshold value of melittins per vesicle is introduced to distinguish the two populations.

We define therefore a minimum number of melittin molecules per vesicle, M_{min}, needed to induce complete calcein release from the vesicle. It is straightforward to calculate the proportion of lysed vesicles since the percentage of release, Pr, corresponds to the probability of finding vesicles with $M \geq M_{min}$. If the probability density function $f(M)$ is known:

$$\Pr(M_{min}, M, \infty) = \int_{M_{min}}^{\infty} f(M)\,dM \quad (3)$$

A similar approach has been applied successfully to describe vesicle leakage induced by GALA [26]. In that case, the peptide distribution on the vesicles was calculated taking into account the partitioning of the peptide between the vesicles and the aqueous environment, since the fraction of bound peptide was evaluated to only 0.645. However, in the case of melittin, it has been shown that, in our conditions, most of the melittin is bound to the lipid bilayer [22,23], leading to simplified calculations. Assuming a random distribution of melittin on the vesicles, the extent of release is expressed by the Gaussian probability density function.

$$\Pr(M_{min}, M, \infty) = \frac{1}{\sigma\sqrt{(2\pi)}} \int_{M_{min}}^{\infty} \exp\left(-\frac{1}{2}\left(\frac{M_{min}-\mu}{\sigma}\right)^2\right) dM \quad (4)$$

where μ is the mean calculated from the melittin/lipid incubation ratio and σ, the standard deviation approximated by $\sqrt{\mu}$. Using our experimental data, we have calculated the value of M_{min} from each melittin/vesicle ratio (providing μ) and percentage of release (Pr); the average M_{min} is 254 ± 46 melittins per vesicle. Considering melittin as a rod of 35 Å long and 12 Å of diameter [38], this value corresponds to less than 4% of the area of a 100 nm vesicle occupied by melittin, assuming that melittin lies parallel to the bilayer surface as suggested by the ATR measurements [39].

Using M_{min}, we have simulated our experimental curve (Fig. 2). The simulated behaviour reproduces the significant features of our data, and provides a rationale for the fact that a certain amount of melittin can be added without inducing release and that the incubation ratio regulates the extent of release. We proposed that melittin distributes randomly onto all the vesicles, and a minimum number of melittins per vesicle, M_{min}, is required to induce release. Thus, for small proportions of melittin, all the vesicles have a number of bound melittins below M_{min}, and no leakage occurs. Increasing melittin/lipid incubation ratio leads to an increased fraction of vesicles exceeding M_{min}. The release is practically total if every vesicle has a number of bound melittin exceeding M_{min}.

The average number of melittin required to induce the release of trapped calcein from a POPC vesicle brings some insights into the lysis mechanism. This large number suggests that the leakage is more likely related to cooperative membrane perturbations than to the formation of a well-defined pore. A single melittin can perturb the lipid bilayer without affecting its impermeability integrity. However, the sum of individual perturbations could lead to a leaky vesicle when a critical number of toxin per vesicle (defined here as M_{min}) is reached. This is consistent with the detergent-like effect of melittin proposed for the changes in bilayers permeability [14,40]. The formation of lesions has been also suggested for other cytotoxic agents. Because of their common features of damage inflicted to plasma membranes and their synergistic effects, a series of agents including Sendai virus, the *S. aureus* α toxin, and components of activated complement, have been proposed to cause membrane lesions instead of the formation of defined channels [4]. Cooperative effect leading to a severe disruption of the membrane has also been proposed for gramicidin S [14,40] and magainin [41].

4. Conclusion

The melittin-induced lysis of phospholipid bilayers occurs by an all-or-none mechanism leading to complete calcein release from individual liposomes. Melittin binding is rapid, and subsequent migration of melittin between vesicles was not observed; thus, the release of material stops after reaching its maximum a few minutes after the addition of the toxin. The percentage of release depends on the molar incubation ratio melittin/lipid. However, it is possible to add a certain amount of melittin to vesicles without detectable release. A new characteristic of melittin revealed in this study is its capacity to distinguish between the intact and lysed vesicles, suggesting that the bilayer properties of these two populations are significantly different. A simple statistical model introduces the concept of a minimum number of melittins per vesicle to induce release from a POPC vesicle. This number is evaluated to 250 melittins per vesicle; this large number suggests that the leakage is more likely due to the toxin molecules perturbing in a cooperative manner the bilayer permeability rather than to the formation of a well-defined pore. Further, we showed that the presence of negative charge density on the surface of phosphatidylcholine bilayers inhibits the lytic power of melittin. The decrease in the potency of melittin is proportional to the increase in negative charge density up to 30 mol% for PA and 50 mol% for POPG. It is proposed to be due to the electrostatic anchorage of the peptide at the bilayer interface.

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